Am J Cancer Res 2023;13(1):1-24
www.ajcr.us /ISSN:2156-6976/ajcr0145134

Review Article

Basic and translational research on carbon-ion radiobiology

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Received July 4, 2022; Accepted November 16, 2022; Epub January 15, 2023; Published January 30, 2023

Abstract: Carbon-ion beam irradiation (IR) has evident advantages over the conventional photon beams in treating tumors. It releases enormous amount of energy in a well-defined range with insignificant scatter in surrounding tissues based on well-localized energy deposition. Over the past 28 years, more than 14,000 patients with various types of cancer have been treated by carbon ion radiotherapy (CIRT) with promising results at QST. I have provided an overview of the basic and translational research on carbon-ion radiobiology including mechanisms underlying high linear energy transfer (LET) carbon-ion IR-induced cell death (apoptosis, autophagy, senescence, mitotic catastrophe etc.) and high radiocurability produced by carbon-ion beams in combination with DNA damaging drugs or with molecular-targeted drugs, micro-RNA therapeutics and immunotherapy. Additionally, I have focused on the application of these treatment in human cancer cells, especially cancer stem cells (CSCs). Finally, I have summarized the current studies on the application of basic carbon-ion beam IR according to the cancer types and clinical outcomes.

Keywords: Carbon-ion irradiation, cancer stem cell, DNA damage, cell death

Introduction

Cancer treatment mainly includes surgical resection, chemotherapy, immunotherapy and radiotherapy [1-3]. Radiotherapy is one of the most common methods to treat tumors, however, conventional photon beam irradiation (IR) enhances tumor migration and invasiveness, thereby promoting tumor recurrence and metastasis of cancer cells [4, 5]. In contrast, the high energy and good dose distribution of heavy ion radiotherapy has resulted in desirable outcomes in various type of tumors, which are resistant to conventional radiotherapy and chemotherapy [6-9]. CIRT is a cutting-edge radiotherapy technique with excellent QOL that is less invasive to the body. From June 1994 to March 2022, more than 14,000 patients with cancer have been treated using CIRT at QST and have achieved promising results [10-12].

Carbon-ion beams have evident advantages over the conventional photon beams. They release enormous amount of energy based on well-localized energy deposition, which can be used to treat various cancer types including deep-sited and radioresistant tumors [13-15]. Numerous studies have been conducted on the mechanisms of CIRT. I have summarized the mechanisms of carbon-ion beam IR-induced cell death, and reports on basic and translational research on the effect of carbon-ion beam IR alone or in combination with DNA damaging drugs, molecular-targeted drugs, miRNA therapeutics and immunotherapy on tumor cell death, cell migration, invasion, metastasis, and various radioresistant cancer stem cells (CSCs).

Carbon-ion beam irradiation (IR) induced cell death mechanisms

IR can induce different cell death modalities, which depends on various factors, including cell type, radiation doses and quality, oxygen tension, TP53 status, DNA repair capacity, cell cycle stages during radiation exposure, and the microenvironment. The major types of IR-induced cell death include such as apoptosis,

autophagy, senescence, mitotic catastrophe and mitotic death, and necrosis [16-21].

Apoptosis is a highly regulated form of cell death with characteristic morphological and molecular features. In the endogenous (mitochondrial) apoptotic pathway, the DNA damage repair mechanism centered on p53, disturbs the balance between pro-apoptotic factors and anti-apoptotic factors, and induces the release of cytoplasm and activated caspase-9 from mitochondria to the [22].

Apoptosis is one of the most important molecular mechanisms of high LET carbon-ion beam IR-induced cell death. Carbon-ion beam IR induces p53-independent apoptosis in lung cancer [23]. Additionally, p53 KO cells were more resistant to X-ray IR than the p53 wild type, while the sensitivity of p53 wild type and p53 KO cells to carbon-ion beam IR was not significantly different. Both X-ray and carbon-ion beam IR predominantly induced the apoptosis of p53 wild type cells but not of p53 KO cells [24]. We also reported that carbon-ion beam alone or in combination with chemotherapy or molecular-targeted drugs can induce apoptosis in different tumor cells [25-28].

Autophagy is the protective mechanism of sequestering damaged or older organelles in vesicles due to lysosomal degradation in response to cellular stress. However, an excessive autophagy response will result in autophagy-induced cell death [26]. Autophagy is a highly regulated pathway involving the ATG gene, the exact mechanism of which is unknown, but is probably caused by IR via the endoplasmic reticulum stress module and the mTOR pathway [26]. Depending on the therapy, autophagy after IR may be a potential treatment mechanism of cell death [17, 20, 29]. Tumor cell autophagy levels increased depending on the LET after high LET carbon ion beam IR via endoplasmic reticulum stress response (UPR) -eIF2α-CHOP-Akt signaling axis [30]. Additionally, we reported that carbon-ion beam IR alone or in combination with chemotherapy or molecular targeted drugs can induce apoptosis/autophagy in pancreatic, colorectal and breast cancer cells [26-28, 31]. RT2 Profiler PCR Array analysis showed that carbon ion beam IR combined with gemcitabine (GEM) remarkably induced the expressions of multiple cell death-related genes [28]. The expression of some apoptotic and autophagy-related genes such as Bax, Bcl2, Beclin1 and ATG7 was significantly induced by carbon-ion beam IR, and was further enhanced when the beam was combined with 5-FU [31].

Senescence refers to the permanent arrest of the cell cycle. IR-induced senescence is caused by various factors including DNA damage, induction of the p53 and pRb pathways that cause cell cycle blockage, or oxidative stress [32-34]. Carbon-ion beam IR induces autophagy, and cellular senescence in a human glioma-derived cell line [35], whereas it impaired mitochondrial function and suppressed telomerase activity via hTERT down-regulation in MCF-7 BC cells [36]. Recently, carbon ion beam IR have been demonstrated to induce premature senescence in p53 wild type cells via the p21-dependent pathway [37].

Mitotic catastrophe is caused by the incomplete mitosis, resulting in mitotic arrest and ultimately regulated cell death or senescence [38]. Mitotic death refers to regulated cell death (usually intrinsic apoptosis) that is driven by mitotic catastrophe [39]. IR leads to mitotic catastrophe through dysfunctional cell cycle checkpoints which induces cancer cells to enter mitosis prematurely with misrepaired DNA [40-42]. Mitotic catastrophe was more efficiently induced by carbon ion beam IR than by the same physical dose of X-rays, whereas apoptosis and senescence were not, suggesting that the correlation of sensitivity to carbon ions and cisplatin is weaker than that of sensitivity to X-rays and cisplatin [43]. In addition, carbon-ion beam IR markedly induced mitotic catastrophe in p53 KO ells, whereas X-rays did not [44]. High LET carbon-ion IR can induce chromosome rearrangements and reproductive cell death due to both the complexity and absolute number of DNA damage clusters [45].

IR induces the immunogenic cell death (ICD) of cancer cells and produce anti-tumor immunity through the release of damage-associated molecular patterns (DAMPs) including calreticulin (CALR) exposure on the cell membrane, adenosine triphosphate (ATP) secretion, high-motility group box 1 (HMGB1) release into the extracellular space, HSP70 and HSP90 exposure, chemokine release and type I interferon (IFN-I) secretion [46, 47]. These IR-induced im-

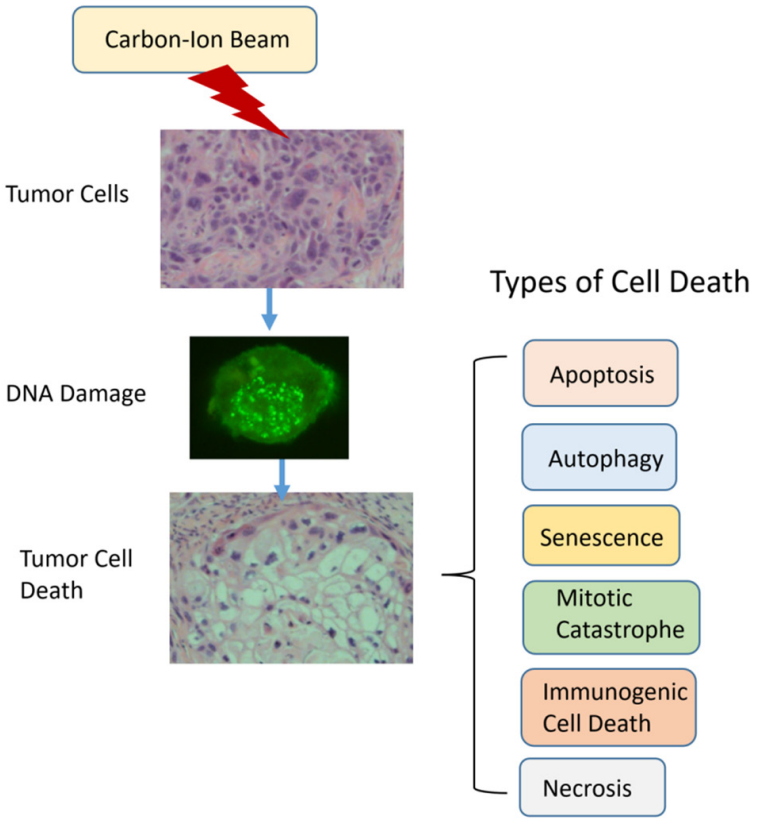

Figure 1. The effect of carbon-ion beam IR on tumor cells. This schematic diagram shows the mechanisms of carbon ion beam IR which can induce complex DNA damage, resulting in induced tumor cell apoptosis, autophagy, senescence, mitotic catastrophe, and necrosis.

munogenic responses result in the uptake of tumor antigens by dendritic cells (DCs) and promote the differentiation of T cells into CD8+ cytotoxic T lymphocyte to activate anti-tumor immunity [48]. A recent report showed that the combination of carbon-ion beam IR and anti-PD-1 antibodies more efficiently triggered hallmarks of ICD, such as CALR exposure, ATP release, HMGB1 efflux, and immunogenicity compared to conventional radioimmunotherapy. Moreover, carbon-ion beam IR and anti-PD-1 antibodies enhanced the infiltration of CD4+ and CD8+ lymphocytes into the tumor bed, resulting in delayed tumor growth, and prolonged survival in melanoma-bearing mice [49, 50].

Altogether, carbon-ion beam IR can induce complex DNA damage and consequently tumor cell death via various pathways such as apoptosis, autophagy, senescence, mitotic catastrophe, ICD and necrosis (**Figure 1**).

Effects of carbon-ion beam IR on CSCs

CSCs, a small subpopulation of cells in the tumor bulk, have self-renewing potential, are resistant to chemotherapy or radiotherapy, and are associated with tumor recurrence and metastasis [51-53]. Thus, eliminate CSCs effectively can ensure high curability. We can identify CSCs using several cell surface markers depending on the tumor types [54-56]. CSCs are likely located in the hypoxic area [57-59]. We hypothesized that carbon-ion beam IR can be used to kill CSCs as it has shown effective treatment of radioresistant hypoxic tumors. We mainly focused on the treatment of colorectal, pancreatic, and BC using carbon-ion beam IR alone or in combination with DNA damaging drugs or molecular targeted drugs [26-28, 31]. **Table 1** presents a list of CSC-related reports from several research groups, including ours [25-28, 60-64]. Other groups have mainly reported the use of carbon-ion beam IR to kill H&N and Glioma, and cervical CSCs [64-73]. In fact, our pioneering study conducted 10 years ago explored the effects of carbon-ion beam IR on colorectal CSCs in vitro and in vivo. In vitro RBE values for CSCs calculated from the D10 levels, ranged from 2.05 to 2.28, whereas an in vivo xenotransplant assay showed an RBE of 3.05 to 3.25, calculated from the slope of the dose-response curve for tumor growth suppression. Immunohistochemistry analysis indicated that CSC markers were significantly suppressed by 30 Gy carbon-ion beam IR [60]. To expand carbon ion treatment applications, we also investigated whether carbon-ion beam IR combined with 5-fluorouracil (5-FU), a DNA damaging drug is superior to carbon-ion beam IR alone in targeting colorectal CSCs in vitro and in vivo. We found that carbon-ion beam IR alone decreased CRC cell viability, whereas it significantly enhanced cell killing effects when combined with 5-FU. Carbon-ion beam in com-

Table 1. List of studies in carbo-ion beam irradiation effects on human cancer stem cells

Energy	LET (keV/um)	Dose (Gy)	Cells	with Drugs	Ref.
(Our group)					
290MeV/n	50	1, 2, 3	Colon cancer cell (HCT116, SW480)	No	[60]
290MeV/n	50	1, 2, 3	Pancreatic cancer cell (BxPc3, MiaPCa2)	No	[61]
290MeV/n	50	1, 2, 3	Breast Cancer cell (MDAMB231, MDAMB453)	Cisplatin	[27]
290MeV/n	50	1, 2, 3	Pancreatic cancer cell (PANC1, PK45)	Gemcitabine	[28]
290MeV/n	13, 50, 78	1, 2, 3	Mesothelioma cell (MESO1, H226)	Cisplatin	[63]
290MeV/n	50	1, 2, 3	Breast Cancer cell (BT474, SKBR3)	Lapatinib	[26]
290MeV/n	50	1, 2, 3	Colon cancer cell (HCT116, HT29)	5-FU	[25]
290MeV/n	50	1, 2, 3	Chondrsarcoma cell (CH-2879)	miR-34a mimic	[64]
290MeV/n	50	1, 2, 3	Pancreatic cancer cell (PANC1, PK45)	miR-200c mimic	[62]
(Other groups)					
11.4, 75MeV/n	33.6, 184	1, 2, 3, 4, 10	Head and Neck cancer cell (SQ20B)	UCN-01 (Chk1 inhibitor)	[65]
290MeV/n	70	1, 2, 3	Glioblastoma (A172, U251, U87)	No	[78]
75MeV/n	33.6	1, 2, 3, 4, 5	Head and Neck cancer cell (SQ20B)	Cetuximab	[71]
75MeV/n	33.6	1, 2, 3, 4, 5	Head and Neck cancer cell (SQ20B, FaDu)	No	[73, 75, 76]
N. P	N. P	1, 2, 3	Glioblastoma (SMA-560, GI261 murine glioma, human primary GSC-enriched cultures (NCH644, NCH441, and T325)	No	[79]
N. P	N. P	1, 2, 3	Glioblastoma (Patients derived)	No	[77]
290MeV/n	50	1, 2, 3	Cervical cancer cell (ME180, CaSki)	No	[195]

N.P, Not Provided.

bination with 5-FU significantly inhibited the spheroid forming capacity of CD133+ cell subpopulations [31].

The effect of carbon-ion beam IR alone on pancreatic CSCs was first reported by Oonishi [61]. The number of spheroids formed from CSCs after carbon-ion beam IR was significantly reduced compared to that of X-ray IR. We examined the combination treatment of carbon-ion beam IR and GEM, a nucleoside analogue that causes termination of DNA synthesis. We found that both colony and spheroid formation abilities were extremely suppressed when carbon-ion beam IR was combined with GEM. A DNA damage and repair assay was performed using the DSB marker γH2AX immunofluorescence staining. Higher and bigger γH2AX foci were formed in the CSC 24 h after treatment with carbon-ion beam IR alone compared to that of X-ray IR, and it was further increased when combined with GEM. In vivo PK45 xenograft tumor control analysis showed that high dose of 30 Gy carbon-ion beam IR alone or relatively low dose of 25 Gy combined with GEM effectively destroyed tumor cells compared to low dose of 15 Gy carbon-ion beam IR or high dose of 30 Gy X-ray combined with GEM, as evidenced by the histopathological features. Immunofluorescent staining for CSC markers clearly indicated that relatively low dose 25 Gy combined with GEM effectively eliminated CSCs [28]. Recent studies have shown that microRNA, a small non-coding RNA molecule (containing about 22 nucleotides) is a promising drug target. MiR-200c, known to act as a tumor suppressor, inhibits tumor growth and metastasis by suppressing the CSCs. Therefore, we investigated how a combination of miR200c and carbon-ion beam IR target pancreatic CSCs. Combining carbon-ion beam IR with the miRNA-200c mimic significantly reduced viability of both non-CSCs and CSCs and reduced their colony and spheroid formation abilities compared to that by carbon-ion beam IR alone or X-ray combined with the miRNA-200c mimic [62].

Triple-negative breast cancer (TNBC) cells lack estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC is an aggressive BC subtype with poor prognosis [74]. The surviving fractions of CSCs and non-CSCs decreased exponentially with increasing doses of either carbon-ion beam IR or X-rays, and the decrease in non-CSCs was significantly higher than that of CSCs. The RBE value calculated at the D10 level for CSCs was calculated to be approximately 2.14, whereas that for non-CSCs was

approximately 1.78. This signifies that carbon-ion beam IR is twice as effectively at killing TNBC CSCs. CDDP, a DNA-damaging antitumor drug, is reported to induce the differentiation of breast CSC and make them easily killable. We found that carbon-ion beam IR combined with CDDP effectively suppressed colony, spheroid formation ability of CSCs. In addition, expressions of the CSC markers CD44 and ESA were almost completely eliminated by carbon-ion beam IR combined with CDDP, whereas X-ray, carbon ion beam, CDDP alone or X-ray combined with CDDP significantly increased the expression of ESA [27].

Tumors with HER2 overexpression tend to be of a higher grade and are more likely to spread, making them more aggressive than other types of BCs [68, 70]. Approximately 10-15% of BC tumors in women are HER2+. HER2+ BCs can be treated with anti-HER2 drugs such as trastuzumab [72]. Lapatinib is dual inhibitor of EGFR and HER2 [66, 69]. Here we examined carbon-ion beam IR alone or combined with Lapa to treat HER2+ BC cells. The proportion of CSCs in BT474 cells was significantly increased after treatment with 2 Gy of X-rays combined with lapatinib or with lapatinib alone, whereas 1 Gy of carbon-ion beam IR combined with lapatinib significantly decreased the proportion of CSCs [26].

High-grade chondrosarcoma exhibits poor prognosis and low survival rate [67]. Recently, the combination of carbon-ion beam IR with miR34 mimic and/or mTOR inhibitor rapamycin has been reported to effectively eradicate high-grade chondrosarcoma CSCs in vitro and in vivo. Administration of tumor cells with miR-34 mimic together with carbon-ion beam IR resulted in a significant decrease in tumor formation [64].

CSCs (side population/CD44(+)/ALDH (high)) from head and neck squamous cell carcinoma (HNSCC) cell line (SQ20B) were more resistant to both photon and carbon ion beam IR compared to non-CSCs. CSCs were capable of an extended G2/M arrest phase in response to photon or carbon-ion beam IR. The combination of UCN-01 (Chk1 inhibitor) and all-trans retinoic acid (ATRA) significantly sensitized CSCs to both photon and carbon ion beam IR [65]. Additionally, carbon-ion beam IR can overcome hypoxic radioresistance of HNSCC associated with DNA repair and significantly inhibited migration and invasion in both the HNSCC cell lines SQ20B and SQ20B/CSCs [73, 75, 76], and the combination with cetuximab significantly inhibited invasion in both populations. In contrast, photon beam IR enhanced SQ20B migration and invasiveness in both populations, while cetuximab only inhibited the migration [71, 73].

Carbon-ion beam IR efficiently eradicated radiation-resistant patient-derived glioma stem cells (GSCs), resulting in growth inhibition and prolonged survival. Carbon-ion beam IR (15 Gy) suppressed tumor regrowth and achieved long-term local tumor control. Fractionated carbon-ion IR further prolonged survival. The enhanced RBE of carbon ion beam IR in vivo was due to its strong anti-angiogenic effect and the eradication of radiation-resistant hypoxic tumor cells. Blocking of the HIF1-α/stromal cell-derived factor 1/CXCR4 axis using carbon ion beam IR reduced the recruitment of microglia and myeloid-derived suppressor cells. As a result, carbon ion IR nullified M2-like immune polarization, promoted CD8+ cell influx, and created an immune-tolerant niche. Carbon-ion beam IR overcomes several central glioma resistance mechanisms by eradicating hypoxia and stem cell-like tumor cells and regulating the glioma niche towards less anti-angiogenic and immunosuppressive conditions [77, 78].

Recent studies on clonogenic survival analysis using photon radiation-resistant GSCs from human primary glioblastoma have shown proton IR revealed RBEs in the range of 0.7-1.20. However, carbon ion beam IR sensitized the photon beam resistant GSC cultures, with an average RBE of 1.87 to 3.44. This effect was partly due to the impaired GSCs to repair carbon ion-induced DNA double-strand breaks, as determined by residual DNA repair lesions [79].

The main mechanism underlying the effective ablation of CSCs by carbon ion beam IR is believed to be the potent induction of complex, irreparable, clustered DNA lesions, especially in combination with DNA damaging drugs that further promote CSC death [26-28, 31, 80]. In addition, carbon ion beam IR disrupts tumor cells in a cell-cycle independent manner and is less affected by oxygen concentration. This

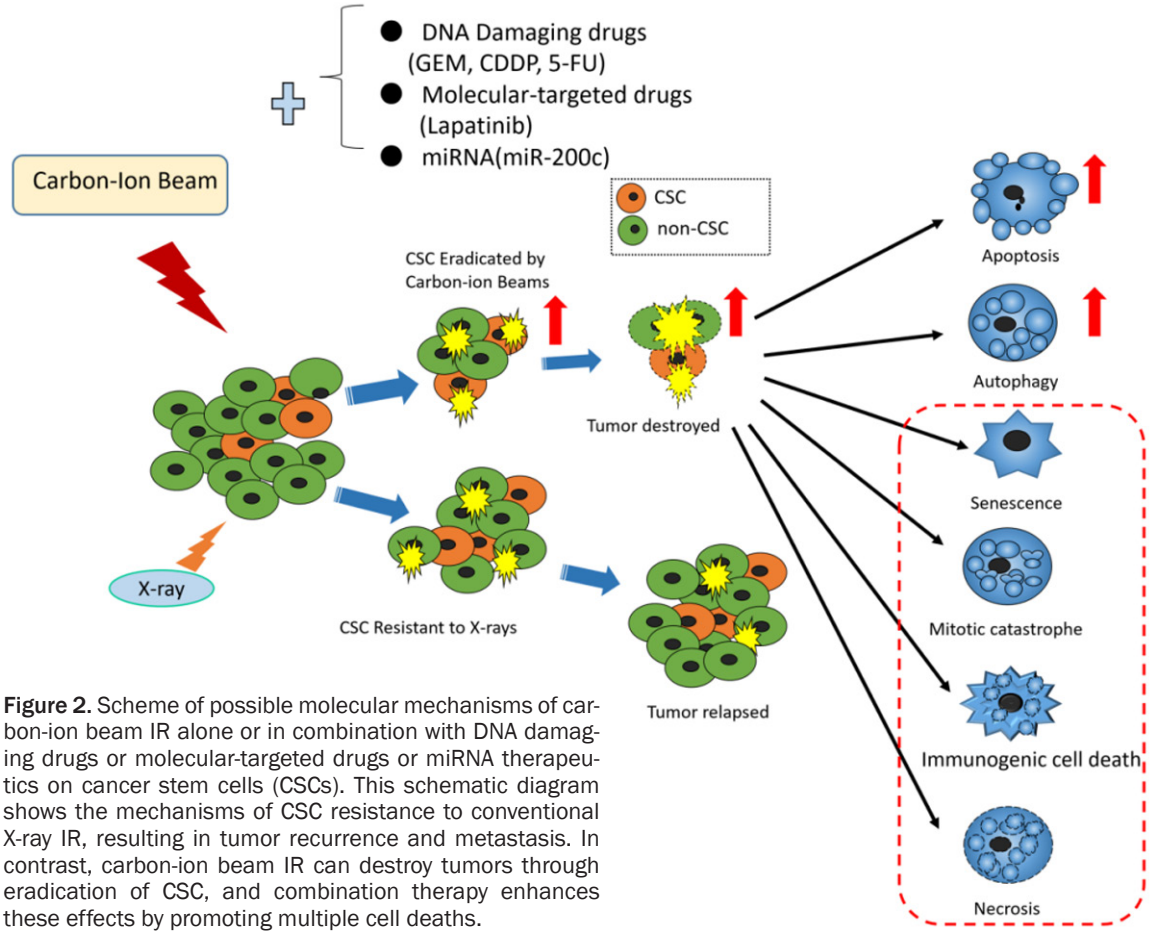

Figure 2. Scheme of possible molecular mechanisms of carbon-ion beam IR alone or in combination with DNA damaging drugs or molecular-targeted drugs or miRNA therapeutics on cancer stem cells (CSCs). This schematic diagram shows the mechanisms of CSC resistance to conventional X-ray IR, resulting in tumor recurrence and metastasis. In contrast, carbon-ion beam IR can destroy tumors through eradication of CSC, and combination therapy enhances these effects by promoting multiple cell deaths.

allows efficient targeting of CSCs that are likely to be located in hypoxic region and are in the G0 or, quiescent phase of the cell cycle.

In summary, carbon-ion beam IR destroys CSCs more effectively compared to conventional X-ray IR and its combination with DNA damaging drugs, molecular-targeted drugs, miRNA therapeutics and immunotherapy synergistically enhances carbon-ion radiosensitivity (**Figure 2**).

Effects of carbon-ion beam IR on tumor cell migration, invasion and metastasis

Conventional photon beam IR may promote tumor cell migration and infiltration through complex mechanisms, such as inducing changes of microenvironment, cell-cell binding, extracellular matrix binding, protease secretion, and epithelial-mesenchymal transition [4, 81, 82]. However, carbon-ion beam IR suppressed the metastatic potential of A549 and EBC-1 cells effectively [83]. Carbon-ion beam IR inhibited glioma and endothelial cell migration [84] and suppressed pancreatic cancer cell migration and invasiveness via Rac1 and RhoA degradation [85]. Both proton and carbon ion beam IR decreased cell migration and invasion in a dose-dependent manner and strongly inhibited matrix metalloproteinase-2 activity. However, lower X-ray IR promoted cell migration and invasion concomitant with up-regulation of alphaV-beta3 integrin expression. Furthermore, carbon-ion beam IR significantly decreased the number of pulmonary metastases *in vivo* [86]. Altogether, the mechanism of tumor cell migration and invasion suppression by carbon ion beam IR is thought to effectively suppress migration and invasion-related proteins such as MMP2 and MMP9 via the effective inhibition of RhoA/Rock1 [85], Pi3K/Akt/mTOR [64, 85] and Ras/MAPK pathways [87-89] (**Figure 3**). However, carbon ion beam IR promotes the migration and invasion of PANC1 cells [90, 91]. Therefore, the effects of carbon ion beam IR on tumor migration and invasion are controversial and require further studies of the associated mechanisms.

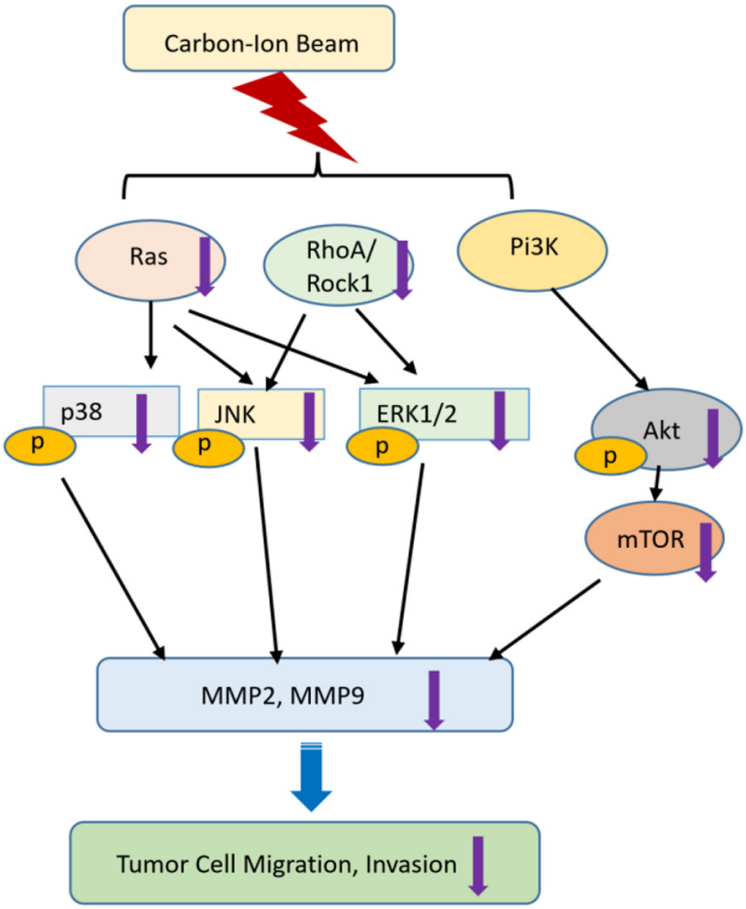

Figure 3. Scheme of possible molecular mechanisms of the suppression of tumor cell migration and invasion after carbon-ion beam IR. Carbon-ion beam IR effectively suppresses migration- and invasion-related proteins such as MMP2 and MMP9 through the inhibition of RhoA/Rock1, Pi3K/Akt/mTOR and Ras/MAPK pathways.

Effects of carbon-ion beam IR in combination with DNA damaging drugs

So far, studies have primarily focused on carbon-ion beam IR monotherapy, which may not give satisfactory results in all types of cancer. The core research problem is how to further enhance the effect of carbon ion beam IR therapy and expand its clinical implications. This requires basic and translational research on combination therapies that can produce maximum synergies [92, 93]. Several anticancer agents exist, for example cytotoxic drugs, which cause DNA damage through the inhibition of DNA synthesis (GEM, 5-FU), DNA cross-linking (cisplatin, CDDP) [94-96].

We have previously reported that carbon ion beam IR destroys pancreatic CSCs more effectively when combined with GEM. Histopathological findings also demonstrated that a relatively low dose carbon ion beam IR (25 Gy) combined with GEM significantly disrupted pancreatic cancer cells [28]. This was the first to show why a combination therapy of carbon ion beams IR and GEM for LAPC achieved promising outcomes at the QST, from the perspective of stem cells [97, 98]. We have also reported that the combined use of the anticancer drug CDDP or 5-FU and carbon-ion beam IR kills refractory BC subtype TNBC stem cells and refractory malignant mesothelioma and colorectal cancer stem cells more effectively than carbon-ion beam IR alone [25, 27, 28, 63]. The possible molecular mechanisms of tumor cell death after carbon-ion beam IR alone or in combination with DNA damaging drugs are shown in **Figure 4**.

Effects of carbon-ion beam IR in combination with molecular targeted drugs

In recent years, numerous molecular-targeted drugs that suppress cancer cells by disabling them at a molecular or genetic level have been developed and they contribute to improving the prognosis of patients [99-102]. The molecular targeted drugs induce apoptosis as well as autophagy and senescence [103-107]. These molecular-targeted drugs can suppress cancer cell proliferation and kill cancer cells more effectively when combined with radiation therapy [108-110]. Combination therapy with PARP inhibitors enhanced the cytotoxic effects of γ-ray IR, and LET13 and LET70 carbon ion beam IR in the human pancreatic cancer cell line MIAPaCa-2 [111] (105). Carbon ion beam IR combined with DNA-PK inhibitor induced more DNA fragmentation in H1299 cells than in A549 cells, indicating that it induces different modes of cell death in a p53-dependent manner [112, 113]. In addition, DNA-PK inhibitor NU7441 radiosensitized NSCLC cells to carbon ion beam via

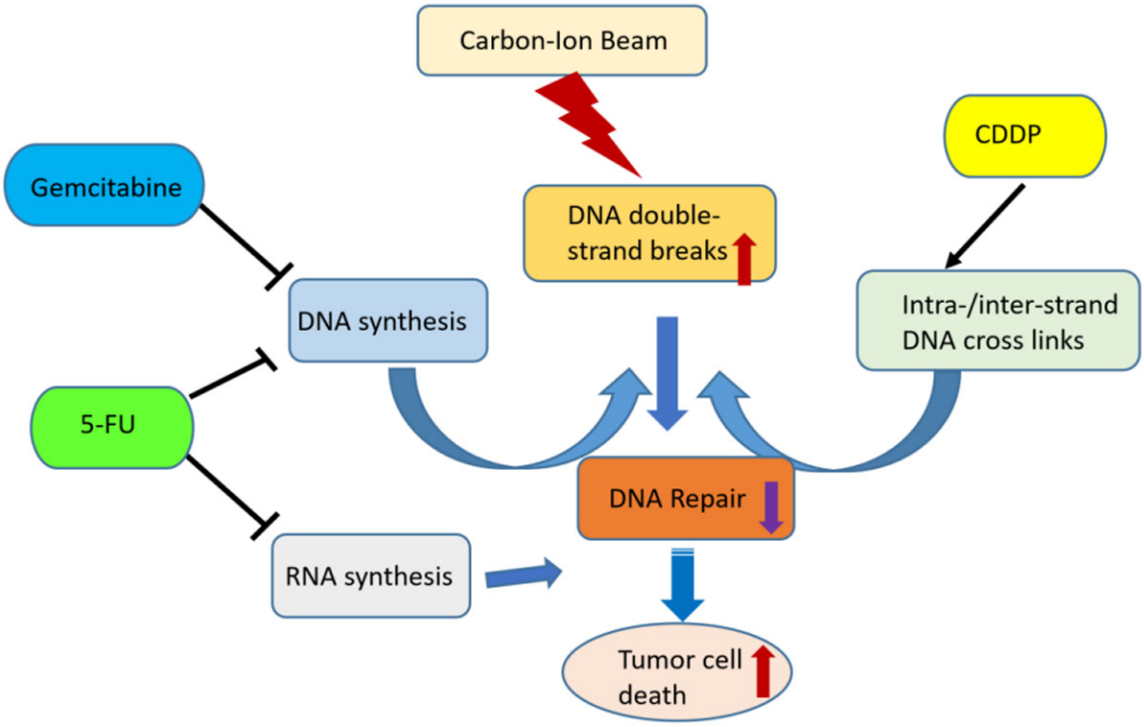

Figure 4. Scheme of possible molecular mechanisms of tumor cell death after carbon-ion beam IR alone or in combination with DNA damaging drugs, such as gemcitabine, CDDP and 5-FU. Carbon-ion beam IR in combination with gemcitabine, CDDP, and 5-FU significantly enhanced DNA double-strand breaks, reduced DNA repair capacity, and resulted in more tumor cell death.

G2/M cell cycle arrest [114]. The novel Hsp90 inhibitor, TAS-116, radiosensitized human cancer cells to both X-rays and carbon ion beam IR by inhibiting two major DSB repair pathways accompanied by marked cell cycle arrest [115]. Furthermore, the combined use of carbon ion beam IR and tyrosine kinase inhibitor lapatinib has a high killing effect on HER2-positive BC stem cells, which are metastatic BC subtypes [26].

Effects of carbon ion beam in IR combination with microRNA (miRNA) therapeutics

MiRNAs, which are small RNA molecules that do not encode proteins, make up 1-5% of the human genome and are expected to interfere with at least 30% of the genes that encode proteins [116-118]. In vitro studies suggest several miRNA candidates that can cause radiation sensitivity. The inhibition of Lin28 by siRNA increased radiosensitivity of AsPc-1 cells through down-regulation of Kras expression [119]. Combination treatment of carbon-ion beam IR and miR-34a mimic significantly inhibited high-grade chondrosarcoma stem cells [64]. MiR-374 overexpression radiosensitized pancreatic cancer cells to carbon ion beam IR [120]. Additionally, miR-29b mimic significantly increased radiosensitivity of osteosarcoma cells to carbon-ion beam IR, and miR-200c mimic enhanced the radiosensitivity of pancreatic cancer cells including CSC to carbon-ion beam IR in vitro and in vivo [62, 121].

Effects of carbon ion beam in combination with immunotherapy

Accumulating evidence shows that radiation can promote immunity through the release of tumor antigens, induction of type I interferon, and modification of the immunosuppressive tumor microenvironment, resulting in an abscopal effect on unirradiated lesions [122-124]. Radiation can stimulate adaptive resistance through the upregulation of PD-L1 expression in tumor cells, and the addition of a checkpoint blockage can overcome this resistance and enhance the generation of abscopal responses [125, 126]. This combination with

radiation is particularly useful in the treatment of immunologically "cold" tumors which are characterized by low levels of T cell infiltration and low mutagenesis [127]. In fact, preclinical data and early clinical trials showed that immune checkpoint inhibitors with RT synergistically enhanced tumor cell killing at local and distant sites via their abscopal effect [128-130]. Interestingly, carbon ion beam IR in combination with anti-PD-L1, anti-CTLA-4, or dendritic cells effectively suppressed the growth of primary and peritoneal tumors, and also reduced lung metastases in mouse models [131-133]. The combination efficiency of carbon ion beam IR with immunotherapy maybe superior compared to that with conventional RT, because carbon ion beam IR can save more circulating immune cells in the blood than X-rays, which is essential for an efficient immune response [134-136].

Taken together, combination therapy of carbon-ion beam IR with DNA damaging drugs, molecular-targeted drugs, miRNA therapeutics and immunotherapy are highly desirable to improve present CIRT and provide more promising outcomes in the future (**Figure 4**).

Basic biological studies and clinical outcomes of carbon-ion beam IR in different types of cancer

Colorectal cancer

Colorectal cancer is the second most common cause of cancer-related deaths in both men and women in the USA [137, 138], and the number of colorectal cancer-related deaths in Japan continues to increase [139]. Carbon-ion beam IR effectively destroyed colorectal CSCs with an RBE 2.1-2.5 in vitro and 3.1-3.4 in vivo [60]. This partially supports clinical data showing carbon-ion beam IR efficiently kills unresectable recurrent rectal cancer with a 44% survival rate [140] (136). Clinical data showed that the 5-year local control and survival rates of patients with locally recurrent rectal cancer after CIRT (73.6 Gy) were 88% and 59%, respectively [141, 142]. To our knowledge, this is the best clinical outcome for advanced recurrent rectal cancer in the world. However, prognosis of recurrent aggressive tumors needs to be further enhanced. To further improve the carbon-ion beam radiocurability, our previous study investigated the effects of combination therapy of carbon-ion beam IR with the anticancer drug 5-FU on colorectal cancer cells. Carbon-ion beam IR combined with 5-FU was superior in targeting colorectal cancer cells including CSCs in vitro and in vivo [31].

Pancreatic cancer

Pancreatic adenocarcinoma is an invasive and fatal malignant tumor, which is currently ranked as the fourth leading cause of cancer-related deaths in the United States and Japan [143]. Carbon-ion beam IR induced apoptosis and G2/M arrest in pancreatic cancer cells [144] and suppressed the migration and invasion of pancreatic cancer cells MiaPaCa2 but increased that in the PANC1 cells [85, 145]. Carbon-ion beam IR can effectively disrupt pancreatic CSCs with severe complex DNA damage, which was more pronounced when combined with GEM [28]. Clinical studies shown that CIRT combined with GEM significantly increased the overall survival (OS) of patients with LAPC, the 2-year OS rate for LAPC is 60% after combination treatment of 55.2 Gy carbon ion beam IR and GEM [97]. Our data can partially explain the molecular mechanisms underlying the effectiveness of CIRT combined with GEM compared to that of CIRT alone [28]. This is a world leading clinical outcome for locally advanced pancreatic cancer treatment compared to any other treatment. However, the present clinical result is still unsatisfactory, because 60% is the 2-year OS and not the 5-year OS. Therefore, there is a need to develop a novel combination therapy to further improve locally advanced pancreatic cancer prognosis. Recently, miR-200c mimic has been shown to have a high potential in enhancing carbon ion beam IR radiosensitivity of pancreatic cancer cells in vitro and in vivo [62]. Combining carbon ion beam IR with the miRNA-200c mimic significantly reduced the viability of both non-CSCs and CSCs and reduced the colony and spheroid formation abilities compared to that in treatment with carbon-ion beam IR alone, or X-ray combined with the miRNA-200c mimic. In vivo data showed that carbon-ion beam IR in combination with the miRNA-200c mimic effectively suppressed xenograft tumor growth with the induction of tumor necrosis and cavitation. Some clinical Phase I trials using miRNAs, like target miR34a and miR155 to treat cancer still underway [146]. We hope that clinical trials

using carbon ion beam IR and miR200c to treat pancreatic cancer will begin in the near future.

Lung cancer

Lung cancer is the leading cause of cancer-related deaths in both men and women, accounting for nearly 25% of all cancer deaths [147, 148]. Carbon ion beam IR decreased HIF-1α levels and drastically delayed xenograft lung tumor growth in vivo [148]. Carbon ion beam IR effectively suppressed migration, invasion and metastasis of human NSCLC cells [83, 149, 150]. Carbon ion beam IR combined with a PARP inhibitor effectively reduced metastatic potential via inhibiting EGFR/Akt/p38/ERK signaling pathway and epithelial-mesenchymal transition [87]. Carbon ion beam IR combined with inhibitors of DNA-dependent Protein Kinase (DNAPKi, M3814) and ATM serine/threonine kinase (ATMi) efficiently eradicated hypoxic lung cancer cells [112]. Carbon ion beam IR combined with MK-1775, an inhibitor of the checkpoint regulatory factor Wee-1, significantly increased γH2AX and mitotic catastrophe in lung cancer cells [151]. This partially supports a clinical result where the 3- and 5-year OS rates of patients with NSCLC after single-fraction CIRT were 91.2% and 81.7%, respectively [152-155]. This clinical result is almost similar to that during surgical treatment, indicating that CIRT is a cutting-edge RT with high QOL. However, patients with a relatively early stage of lung cancer are currently treated using CIRT alone. Therefore, development of a novel combination therapy to expand the clinical implications of CIRT including that in late-stage lung cancer treatment warrants further studies.

Liver cancer

Primary liver cancer is the sixth most common cancer and the fourth leading cause of death from cancer worldwide. The 5-year relative survival rate of liver cancer patients was less than 20% in the USA and Japan [156, 157]. RBE-values of hepatocellular carcinoma (HCC) cells were in the range of 2.1-3.3 and 1.9-3.1 for carbon-ion and oxygen-ion beam IR, respectively [158]. Temsirolimus and GEM with photon IR showed additive cytotoxicity in HCC cell lines, whereas independent toxicities were achieved by combining carbon ion beam IR with these substances [159]. Metformin effectively enhanced the therapeutic effect of radiation at a wide range of LET, particularly carbon ion beam IR [160]. Clinical data showed that the local-control and OS rates in patients with HCC at 3, and 5 years were 91.4% and 50.0%, and 90.0% and 25.0%, respectively after CIRT [161-164]. However, early-stage liver cancer is currently using CIRT alone, because of the lack of effective combination therapy for advanced liver cancer warranting further basic and translational studies.

Head and neck cancer

Head and neck cancer is a term used to describe various malignancies that occur in or around the throat, larynx, nose, sinuses, and mouth. Head and neck cancers account for less than 5% of all cancers for less than 3% of all cancer deaths in the United States [165]. Most head and neck cancers are derived from the mucosal epithelium of the oral cavity, pharynx, and larynx and are collectively known as the head and neck squamous cell carcinoma (HNSCC). Overall and net survival was 26.3% and 41.4% across the HNC site, respectively [165]. Carbon ion beam IR effectively suppressed HNSCC cell and CSC migration/invasion in vitro [65, 71]. The combined effect of photon/hypoxia resulted in synergistic and early HIF-1α expression in both HNSCC CSCs and non-CSCs, which is rarely observed after carbon ion beam IR treatment [73]. In addition, carbon ion beam IR has the potential to overcome the radiation resistance of HNSCCs associated with DNA repair, regardless of the hypoxic microenvironment, especially in CSCs [75, 76]. Clinical studies have shown that CIRT for HN adenoid cystic carcinoma (ACC) results in 2-year overall survival, progression-free survival, and local control rates of 94%, 68%, and 88%, respectively; and the estimated 5-year overall survival, progression-free survival, and local control rates were 74%, 44%, and 68%, respectively [166-170]. However, this is still not satisfactory for some types of HNC especially for mucosal melanoma or adenoid cystic cancer treated by CIRT alone, necessitating basic and translational studies.

Sarcoma

Sarcoma is a general term used for a wide variety of cancers that affect in bone and soft (also called connective tissue) tissue (soft tissue sar-

coma). There are more than 70 types of sarcomas [171]. Treatment of sarcoma depends on the type, location, and other factors of the sarcoma. Osteosarcoma is a type of bone cancer that begins in the cells of bone. Treatment usually includes chemotherapy, surgery, and sometimes radiation therapy [172]. The RBE values of osteosarcoma cells at D10 levels for carbon-ion beam IR at the position of plateau, proximal peak, midpeak, and distal peak within the SOBP were 1.71, 2.48, 2.63, and 3.47, respectively [173]. Carbon ion beam IR enhances the antitumor effect of dual immune checkpoint inhibition therapy (anti-PD-L1 and anti-CTLA-4 antibodies) on both local and distant sites of mouse osteosarcoma [133]. Carbon ion beam IR accelerated reoxygenation of mouse fibrosarcoma [174]. Carbon ion, proton, iron beams, and X-ray caused different bystander signaling in chondrosarcoma cells [175]. CIRT improved the prognosis of unresectable adult bone and head and neck soft tissue sarcoma, with 3-year local control and overall survival of 91.8% and 74.1%, respectively [176]. The 5-year local control, overall survival, and disease-free survival rates of unresectable sacral chordoma after CIRT were 77.2%, 81.1%, and 50.3%, respectively [177, 178]. However, there is still a need to further improve the prognosis of different sarcomas, especially that of aggressive osteosarcoma. The combination carbon ion beam IR with zoledronic acid [179] or miR-29b or miR-34 mimic significantly enhanced carbon ion beam radiosensitivity in osteosarcoma and chondrosarcoma cells [64, 121]. In connection with our basic and translational data, there is hope for future bench-to-bedside research on combination therapies involving CIRT.

Prostate cancer

Prostate cancer is the most common cancer and the second most common cause of cancer death among men in the United States. Treatment of prostate cancer includes active surveillance, surgery, radiation therapy, hormone therapy, chemotherapy, and immunotherapy. The 5-year and 10-year relative survival rates for prostate cancer is approximately 99% and 95%, respectively [180]. Downregulation in several genes involved in the cell cycle and cell motility has been reported after carbon ion beam IR [181]. Carbon ion beam IR reduced radiation response heterogeneity between different prostate cancer cell types, independent of fractionation, compared to photon beams [182]. Clinically overall outcomes of prostate cancer after CIRT showed that the 5-year biochemical recurrence-free survival in low-risk, intermediate-risk, and high-risk patients was 92%, 89%, and 92%, respectively. The 5-year cause-specific survival in low-risk, intermediate-risk, and high-risk patients was 100%, 100%, and 99%, respectively [183-185]. A recent report showed that a DNA repair enzyme PNKP inhibitor radiosensitized prostate cancer cells to carbon ion beam [186], suggesting that CIRT combined with a PNKP inhibitor may further improve prostate cancer outcomes.

Cervical cancer

Cervical cancer is the third most common malignant tumor in women worldwide and continues to be a major cause of cancer-related deaths in women in developing countries [187]. Cervical cancer is treated with surgery, radiation therapy, and chemotherapy. The 5-year survival rate for cervical cancer is 66%. However, survival rates may vary depending on factors such as race, ethnicity, and age [188]. Carbon ion beam IR induces both the caspase-dependent and caspase-independent pathways of apoptosis, and significantly reduced MMP-2 and MMP-9 activities when combined with PARP1 depletion in HeLa human cervical cancer cells [189]. The proliferating cell population (pMI) is a powerful prognostic factor in patients with squamous cell carcinomas of the cervix treated with CIRT [190]. CIRT up-regulated PD-L1 expression a critical immune checkpoint ligand, in both in human cervical cancer cells and clinical specimens and reflected better prognosis [191]. Diallyl disulfide, a crushed garlic extract, enhanced the radiosensitivity of cervical cancer cells to carbon ion beam IR [192]. Carbon-ion beam IR can reduce the radiation resistance properties resulting from cervical tumor hypoxia [193]. Clinical data showed that carbon ion beam IR combined with CDDP improved prognosis of patients with advanced uterine cervical cancer [194, 195]. Recently, it was reported that knock down of Notch signaling factor fused toes homolog (FTS) enhanced the killing effects of carbon ion beam IR on cervical CSCs [196]. This finding may help improve cervical cancer treatment with CIRT.

Melanoma

Melanoma is a serious form of skin cancer that begins in the melanocytes. Surgery is the most

common treatment for melanoma, but its need depends on the extent of cancer progression. The 5-year survival rate from the time of initial diagnosis is 93% [197]. Carbon-ion beam IR significantly downregulated cell cycle-related genes but up-regulated p53 target genes, and induced G2/M arrest [198]. Carbon ion beam IR abolished Lin28B-induced X-ray resistance of melanoma cells [199]. Clinical data showed that the 2-year overall survival and local control rates for head and neck mucosal melanoma after CIRT were 69.4% and 83.9%, respectively [168]. CIRT combined with chemotherapy (dacarbazine, nimustine, and vincristine) resulted in excellent local control, and achieved a 3-year OS, and had a PFS rate of 49.2% and 37.0% respectively, for advanced mucosal melanoma [200, 201]. However, as shown above, the prognosis of melanoma treated by CIRT is still unsatisfactory and therefore the development of a novel combination therapy is necessary. Based on a recent report that carbon ion beam IR can boost anti-tumor immune responses by inhibiting myeloid-derived suppressor cells in melanoma-bearing mice [202], a combination of CIRT and immunotherapy may be a powerful tool to treat melanoma.

Breast cancer

BC has already surpassed lung cancer to become the most common cancer in the world [203]. Although the overall 5-year relative survival rate for BC is high (~90%), BC has the second highest number of cancer deaths after lung cancer in women. Most women with stage I, stage II, and stage III BC are treated with surgery and subsequent radiation therapy [204]. There are several types of BC surgery, including mastectomy, breast-conserving surgery, lymph node resection, and breast reconstruction [205]. High LET carbon ion beam IR can accurately identify the target tumor site without significant scattered radiation, making it suitable for early-stage treatment of BC and for avoiding surgical resection [206]. Carbon-ion beam IR combined with hedgehog pathway inhibitor GANT61 effectively decreased BC cell migration [93]. Clinical trials have already shown that 13 of 14 total patients with stage I BC treated with CIRT maintain a complete response with excellent cosmetic results [207]. However, some subtypes such as TNBC and HER2 positive BC are very aggressive with poor prognosis [208, 209]. In order to expand the application of CIRT, development of a new combinational treatment is necessary. Based on our previous reports that show carbon-ion beam IR combined with the anticancer drug CDDP or the molecular-targeted drug lapatinib can effectively suppress triple negative breast CSCs and HER-positive breast CSCs [26, 27], it is worthwhile to challenge more severe subtypes of BC in the near future.

Glioblastoma

There are several types of gliomas, including astrocytomas, oligodendrocytes, glioblastomas (GBMs), and diffuse gliomas. Among them, GBM is the most common and makes up 40% of all brain tumors [210, 211]. The average survival time is 12-18 months. Only 25% of patients with GBM survive for 1 year or longer, and only 5% of the patients survive for 5 years or longer [212]. Carbon ion beam IR inhibited certain central gliomas by eradicating hypoxic and stem cell-like tumor cells and adjusting the glioma niche to a state of less anti-angiogenesis and immunosuppression [77]. Carbon ion beam IR induced caspase-independent and p53 independent apoptosis in the glioma cells via the PARP-1/AIF signaling pathway [213]. Carbon ion beam IR inhibited glioma cell migration and induced apoptosis, autophagy, and cellular senescence in human glioma cells [79, 214-217]. Clinical data showed a potential benefit of carbon ion beam IR in patients with high-grade gliomas [218, 219]. However, the outcome of glioma after CIRT administration is still unsatisfactory, and there is a need to develop a new and effective strategies to overcome this highly malignant tumor.

Others

Malignant pleural mesothelioma (MPM) caused by asbestos exposure is extremely aggressive and has a long latency period. MPM is a typical refractory cancer whose 5-year survival rate is still less than 10%. Chemotherapy is the only treatment for mesothelioma that has been proven to improve survival in randomized and controlled trials [220]. We have previously reported that RBE values at the 73 keV/μm and 13 keV/μm portion of carbon ion beam IR were estimated as 2.82-2.93 and 1.19-1.22 at D10 level relative to that of X-ray IR, respectively. Carbon ion beam IR combined with CDDP has

Table 2. Representative clinical outcomes after CIRT alone or with drugs at QST

Cancer Type	Dose (Gy/RBE)	Combination Therapy	Outcome	Possible Combination Therapy (Based on our studies)
Colorectal Cancer (Ref. [139-141])	73.6 Gy/RBE	Alone	Recurrent Rectal ~ 5y Local Control (LC) Rate 88% 5y Overall Survival (OS) Rate 59%	5-FU (Ref. [31])
Pancreatic cancer (Ref. [96, 97])	55.2 Gy/RBE	Gemcitabine	Locally Advanced ~ 2y LC Rate 83% 2y OS Rate 60%	miR-200c (Ref. [62])
Lung Cancer (Ref. [152-155])	72.0 Gy/RBE	Alone	Stage T1-2 3y OS Rate 91.2% 5y OS Rate 81.7% Locally Advanced NSCLC 2y LC Rate 80.3% 2y OS Rate 58.7% 2y PFS Rate 40.2%	
Liver Cancer (Ref. [161-164])	45.0 Gy/RBE	Alone	HCC 3y LC Rate 91% 3y OS Rate 67% 5y LC Rate 91% 5y OS Rate 45%	Metformin (Ref. [160])
Head & Neck Cancer (Ref. [166-170])	57.6-64.0 Gy/RBE	Alone or Dacarbazine	2y LC Rate 83.9%; 2y OS Rate 69.4%; 5y LC Rate 75% (melanoma); 73% (adenoid cystic); 61% (papillary, squamous); 24% (sarcoma) 5y OS Rate 35% (melanoma); 68% (adenoid cystic); 56% (papillary, squamous)	
Sarcoma (Ref. [177, 178])	52.8-73.6 Gy/RBE	Alone	1y LC Rate 88% 1y OS Rate 73% 3y LC Rate 82% 3y OS Rate 46%	zoledronic acid (Ref. [179]), miR29b (Ref. [120]), miR34a (Ref. [64])
Prostate Cancer (Ref. [182-184])	57.6 Gy/RBE	Alone	5y Biochemical relapse free (bRF) Rate 89.7% 5y OS rate 95.2%	
Cervical Cancer (Ref. [193, 194])	68.0-74.4 Gy/RBE	Cisplatin	Locally Advanced 2y LC Rate 71% 2y OS Rate 88% 2y PFS Rate 56%	
Melanoma (Ref. [199, 200])	57.6-64.0 Gy/RBE	Alone	Gynecological: 2y LC Rate 71%, OS 53%, PFS 29% Oral mucosal: 5y LC 89.5, OS 57.4%, PFS 51.6%	
Breast Cancer (Ref. [206])	52.8-60.0 Gy/RBE	Alone	Stage I 14 cases median follow up 61 months	TNBC: Cisplatin (Ref. [27]) HER2+: Lapatinib (Ref. [26])
Glioblastoma (Ref. [217, 218])	16.8-24.8 Gy/RBE	Alone	Median OS 17 months Median Progression Free Survival (PFS) 14 months	
Others				
Esophagus Cancer (Ref. [222])	28.8-36.8 Gy/RBE	Alone	Preoperative CIRT Stage I 5y OS 61%, Stage II 77%, Stage III 29%	
Renal Cancer (Ref. [226])	64-80 Gy/RBE	Alone	Stage I-IV 5y LC Rate 94.1% 5y PFS 68.9 5y OS Rate 89.2%	

superior potential to kill MPM cells including CSCs via enhancing apoptosis [63]. We have installed a compact rotating gantry for carbon ions using superconducting magnets that makes it possible to treat tumors of various complex shapes including MPM. Esophageal cancer is an aggressive disease that ranks sixth in *cancer-related death* worldwide [221]. Carbon ion beam IR induced apoptosis in human esophagus squamous cell carcinoma (ESCC) in a dose-dependent manner, significantly reducing cell viability and tumor metastasis [89, 222]. Clinical data showed that the 1-year, 3-year, and 5-year overall survival rates for patients with stage I ESC after CIRT in cases were 91%, 81%, and 61%, that for stage II is 100%, 85%, and 77%, and that for stage III is 71%, 43%, and 29%, respectively [223]. Recently, it has shown that cyclic hydroxamic-acid-containing peptide 31, a histone deacetylase inhibitor (HDACI), enhanced the radiosensitivity of ESCCs to carbon ion beam IR [224]. This finding may provide a possible carbon ion radiosensitizer for ESCC treatment.

Renal cell carcinoma (RCC) is a heterogeneous and most common kidney cancer (> 90%) derived from the renal epithelium [225]. Currently, more than 50% of patients treated for stage I renal cell carcinoma may be cured, but the outcome of stage IV patients is very poor [226]. The long-term outcomes of the RCC after CIRT showed that the LC, DFS and OS rates at 5 years were 94%, 68.9% and 89.2%, respectively [227]. This study suggests that CIRT is a safe treatment option even in inoperative cases, though more promising combination therapies need to be developed.

Representative clinical outcomes of various cancer types treated with CIRT alone or in combination with drugs at QST, and the potential future combination therapy based on our study is summarized in **Table 2**.

Conclusions and future perspectives

Here, I have overviewed basic and translational research on carbon-ion radiobiology, mainly focusing on multi-cell death induction and molecular mechanisms of the high radiocurability induced by carbon ion beam IR alone or in combination with DNA damaging drugs or molecular-targeted drugs, miRNA therapeutics and immunotherapy. Additionally, I have focused on the application of these treatments for targeting human cancer cells including CSCs *in vitro* and *in vivo*. This review will help understanding the advantages of CIRT and will provide a basis for the development of novel combination treatment in the future.

Disclosure of conflict of interest

None.

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